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Ligands for ErbB-family receptors encoded by a neuregulin-like gene

Han Chang*, David J. Riese II†, Walter Gilbert‡, David F. Stern§ & U. J. McMahan*

* Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305-5401, USA
Departments of † Pathology, § Pathology and Biology, Yale University School of Medicine, New Haven, Connecticut 06340, USA
‡ Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138, USA

Neuregulins (also called ARIA[1], GGF[2], heregulin[3] or NDF[4]) are a group of polypeptide factors that arise from alternative RNA splicing of a single gene. Through their interaction with the ErbB family of receptors (ErbB2, ErbB3 and ErbB4), neuregulins help to regulate cell growth and differentiation in many tissues[5–7]. Here we report the cloning of a second neuregulin-like gene, neuregulin-2. The encoded product of the neuregulin-2 gene has a motif structure similar to that of neuregulins and an alternative splicing site in the epidermal growth factor(EGF)-like domain gives rise to two isoforms (α and β). Northern blot and *in situ* hybridization analysis of adult rat tissues indicate that expression of neuregulin-2 is highest in the cerebellum, and the expression pattern is different from that of neuregulins. Recombinant neuregulin-2β induces the tyrosine-phosphorylation of ErbB2, ErbB3 and ErbB4 in cell lines expressing all of these ErbB-family receptors. However, in cell lines with defined combinations of ErbBs, neuregulin-2β only activates those with ErbB3 and/or ErbB4, suggesting that signalling by neuregulin-2 is mediated by ErbB3 and/or ErbB4 receptors.

ErbB2, ErbB3 and ErbB4[8–10] are members of a subfamily of receptor tyrosine kinases that also includes the EGF receptor (EGFR). Signalling through ErbB family receptors is important for regulating cell proliferation and differentiation in many tissues[5–7], and deregulation of these signalling pathways is implicated in a variety of cancers[11]. Although it has been demonstrated that neuregulins can activate ErbB2/3/4 receptors through direct or indirect interaction[12,13], additional ligands for ErbB-family receptors may exist[14–16]. We used a polymerase chain reaction (PCR) based strategy to search for neuregulin-related sequences in an adult rat cerebellum complementary DNA library and have identified a new neuregulin-like gene[17], neuregulin-2.

Figure 1 shows the deduced amino-acid sequence of neuregulin-2β, derived from a composite of two overlapping cDNA clones. This composite contains an open reading frame (ORF) encoding a 754-amino-acid protein. Sequence analysis revealed four structural motifs: a putative signal sequence, a C2-type immunoglobulin-like (Ig-like) domain[18], an EGF-like domain (residues 252–297) with its six characteristic cysteines[19], and a putative transmembrane domain (which separates the whole sequence into a 315-residue extracellular domain and a 414-residue cytoplasmic domain). Another neuregulin-2 cDNA clone, with an extra 77-base-pair (bp) exon inserted between the fourth and fifth cysteine residues of the EGF-like domain, encodes an alternatively spliced variant of neuregulin-2 with a different EGF-like domain (see Supplementary Information). This neuregulin-2 isoform also lacks a transmembrane domain, because the insertion of the extra exon causes a frameshift in the downstream sequence and the termination of the ORF 33 amino acids downstream of the EGF-like domain. Neuregulin-2 molecules having two variant EGF-like domains are termed neuregulin-2α and neuregulin-2β, respectively. The neuregulin gene also has a similar alternative splicing site that gives rise to the α- and β-subtypes of neuregulins[3,5,20], although neuregulin-2α and neuregulin-2β are about equally distant from neuregulin-α or from neuregulin-β. Moreover, there is another alternative splicing site in the cytoplasmic domain of neuregulin-2 in other neuregulin-2 cDNA clones (data not shown), corresponding to the a/b/c-tail splicing site in the neuregulin gene[20]. Therefore, neuregulin-2 and neuregulin not only have similar sequences, they also have similar gene structures.

A protein-database search revealed that the neuregulin-2 proteins are most similar to neuregulins (and to heregulin-β1 among the isoforms of the neuregulins). Overall neuregulin-2β shares 45% identity with heregulin-β1 (ref. 3) and 40% with GGFII (ref. 2). Apart from the N terminus of neuregulin-2β, the similarity between neuregulin-2β and heregulin-β1 extends through their entire sequence (Fig. 2). On the other hand, the N terminus of neuregulin-2β has significant identity to that of GGFII (43%) (Fig. 2). The

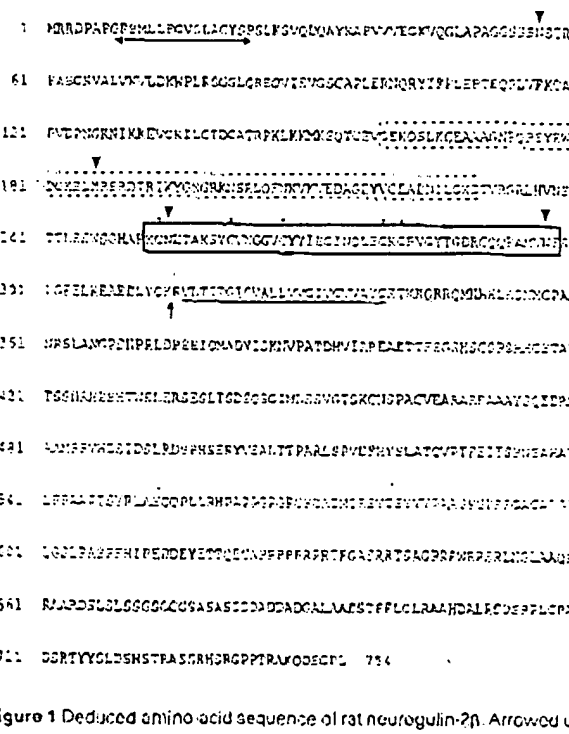

Figure 1 Deduced amino-acid sequence of rat neuregulin-2β. Arrowed underline marks the putative signal sequence. The immunoglobulin-like domain is outlined by a dashed box. Solid frame surrounds the EGF-like domain; the six cysteines characteristic of this domain are indicated by asterisks. Potential *N*-glycosylation sites are marked with arrowheads. The putative transmemorane region is underlined. An arrow points to the potential proteolytic site.

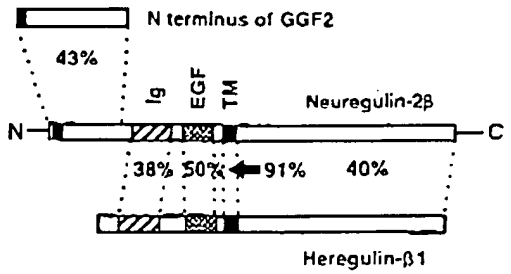

Figure 2 The motif structure of the neuregulin-2β and its similarity to selected members of neuregulins. The percentage similarity is calculated from the amino-acid sequence alignment of neuregulin-2β, heregulin-β1 (human)[3] and the N terminus of GGFII (human)[2]. Black boxes, potential signal sequences; Ig, immunoglobulin-like domains; EGF, EGF-like domains; TM, transmemrane domains.

EGF-like domain [illegible]

most similar region between neuregulin-2β and heregulin-β1 is the transmembrane domain (91% identity) and adjacent sequence. Highly conserved regions also exist in the cytoplasmic tails of neuregulin-2β and heregulin-β1, indicating that the cytoplasmic domains may be important biologically. Relatively high conservation between neuregulin cytoplasmic tails from distant vertebrate species has been noted before[1]. As the EGF-like domain of neuregulins is sufficient for receptor binding and for stimulating cellular responses[5], we compared the EGF-like domain of neuregulin-2 molecules with other EGF-like motifs. Among the known EGF-like motifs, the EGF-like domain of neuregulin-2 is most similar to that of the neuregulins (48% identity between terminal cysteines in the case of heregulin-β1). Second to neuregulins is the rat epidermal growth factor, with 43% identity between terminal cysteines.

To determine the size and tissue distribution of neuregulin-2 mRNAs, northern blot hybridization with poly(A)$^+$ RNA was carried out using a probe spanning the EGF-like domain plus the immunoglobulin-like domain (Fig. 3A). Among the adult rat tissues examined, neuregulin-2 transcripts were most abundant in neural tissues (brain and spinal cord) and lung. A separate experiment with total RNA samples shows that the cerebellum has the highest concentration of neuregulin-2 transcripts compared to other parts of the brain and other adult tissues (data not shown). Three bands were seen in brain samples (Fig. 3A): a prominent band of 3 kilobases (kb), and two additional bands of 3.8 and 6 kb. Only the 3- and 3.8-kb transcripts were detected in spinal cord and lung samples. This pattern of three principal transcripts has also been found for the neuregulin gene, but at the sensitivity of the northern blot, the tissue distribution of neuregulin-2 transcripts in adult rat seems to be more restricted than that of neuregulins[3,4].

We also characterized neuregulin-2 expression by *in situ* hybridization with several probes. In adult rat brain sections, the highest expression was detected in the cerebellum (in the Purkinje cell layer and the granule cell layer) and in the dentate gyrus of the hippocampus (Fig. 3B). Labelled cells were also found in the olfactory bulb (data not shown). This expression pattern seems to be distinct from that of neuregulins, because no hybridization signal for neuregulins is observed in Purkinje cells and very little in the granule cell layer[1]. We investigated the expression pattern of

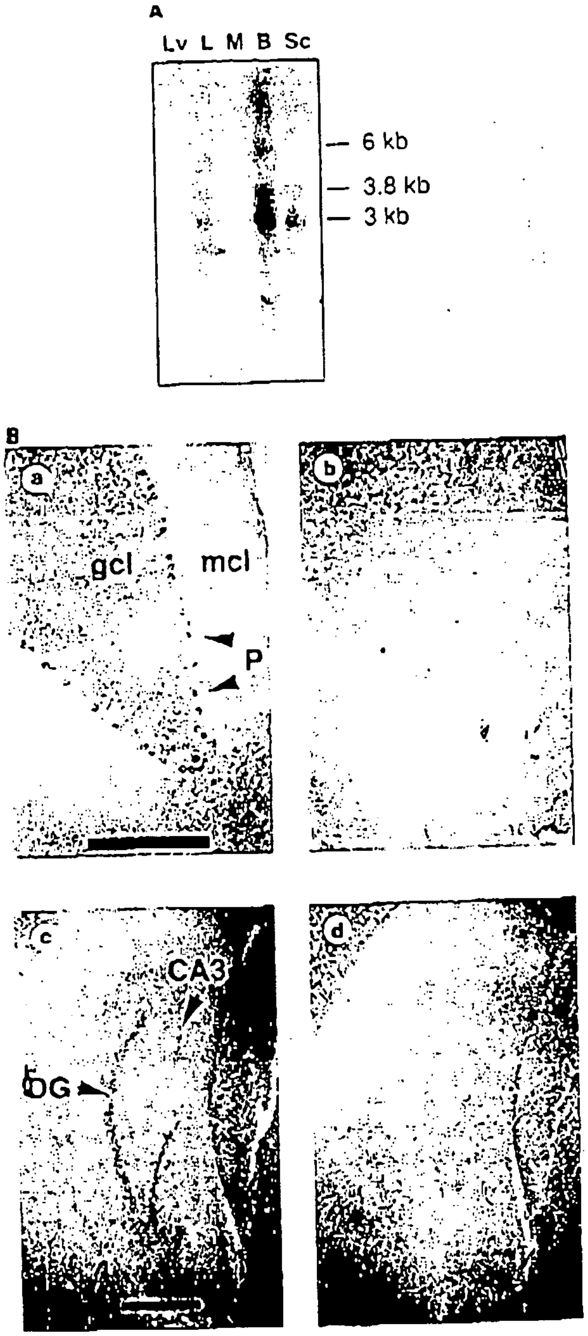

Figure 3 Expression of neuregulin-2 transcripts in adult rat tissues. **A**, Northern blot analysis using poly(A)$^+$ RNA samples; approximately 2 μg poly(A)$^+$ RNA was loaded in each lane. The three bands detected (3, 3.8, 6 kb) are indicated. Lv, liver; L, lung; M, skeletal muscle; B, brain; Sc, spinal cord. **B**, *In situ* hybridization of adult rat brain parasagittal sections with a digoxingenin-labelled cRNA probe spanning the EGF-like and Ig domains. **a**, Neuregulin-2 transcripts were detected in Purkinje cells (P) and in the granule cell layer (gcl) in the cerebellum, but not in the molecular cell layer (mcl); scale bar, 0.4 mm. **b**, Adjacent section hybridized with a sense control probe. **c**, In the hippocampus, neuregulin-2 transcripts were only detected in the dentate gyrus (DG) but not in the CA1–CA3 area; scale bar, 0.8 mm. **d**, Adjacent section hybridized with the sense control probe.

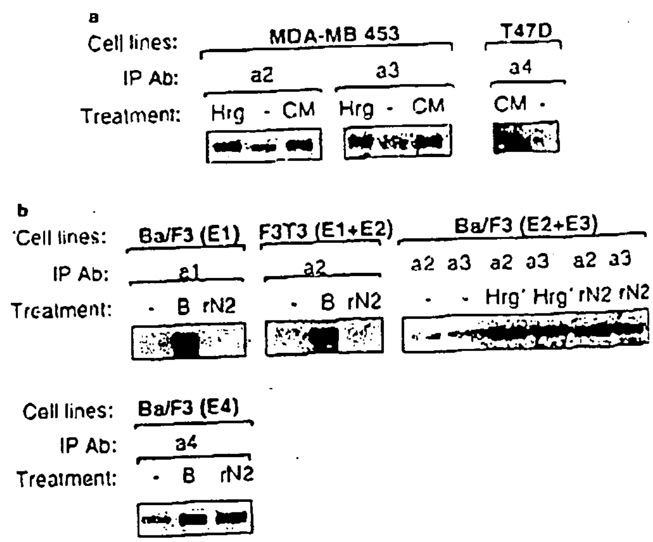

Figure 4 Recombinant neuregulin-2β protein induces tyrosine-phosphorylation of ErbB-family receptors through ErbB3 and ErbB4. **a**, Neuregulin-2β induces tyrosine phosphorylation of ErbB2, ErbB3 and ErbB4 in MDA-MB 453 and T47D cell lines. **b**, Neuregulin-2β signalling through ErbB3 and ErbB4 receptors. rNRG-2β was tested on cell lines transfected with defined ErbB-family receptors. Only, cells with ErbB3 and/or ErbB4 receptors were activated. E1, EGF receptor; E2, ErbB2; E3, ErbB3; E4, ErbB4. Immunoprecipitating antibodies (IP Ab). a1, anti-EGF receptor; a2, anti-ErbB2; a3, anti-ErbB3; a4, anti-ErbB4. B, betacellulin; Hrg', heregulin-β1 EGF-like domain; rN2, rNRG-2β (EGF-like domain of neuregulin-2β); CM, neuregulin-2β-conditioned medium; −, negative control.

neuregulin-2 during embryogenesis by whole-mount *in situ* hybridization with the same probes. We did not detect any signal in E9.5–E10 mouse embryos (data not shown), indicating that none or very little neuregulin-2 is expressed in embryos at those developmental stages. Therefore neuroregulin-2 is unlikely to be the 'missing ligand' for ErbB4 receptors[15].

The structural similarity between neuregulin-2 and neuregulins suggests that the neuregulin-2 proteins may also function as ligands for ErbB family receptors. To test this, we expressed a large portion of neuregulin-2β (including all of the extracellular domain and part of cytoplasmic domain) in CHO cells. As the sequences around the putative proteolysis sites are highly conserved between neuregulin-2 and neuregulins, a soluble form of neuregulin-2β protein should be released from its precursors to the culture medium, as in the case of neuregulins[1–4]. We treated cells expressing ErbB family receptors (MDA-MB453 and T47D breast cancer cell lines)[2,27] with conditioned medium from stably transfected CHO cells. As shown in Fig. 4a, ErbB2, ErbB3 and ErbB4 receptors were activated by neuregulin-2β-conditioned medium. But as ErbB family receptors can form ligand-induced heterodimers, the activation of ErbB2/3/4 receptors could be due to direct or indirect interaction with neuregulin-2β. We also expressed the EGF-like domain (amino acids 240–305; Fig. 1) of neuregulin-2β in bacteria and produced a refolded neuregulin-2β protein fragment (rNRG-2β) from inclusion bodies. rNRG-2β can activate ErbB-family receptors in our breast-tumour cell lines (data not shown), suggesting that like neuregulins, the EGF-like domain is the functional domain for activating ErbB-family receptors.

To determine which of the ErbB family receptors is involved in neuregulin-2β signalling, we tested rNRG-2β on cell lines expressing defined combinations of ErbB receptors. We did not detect rNRG-2β activation of EGF receptors in the Ba/F3 (EGFR) cell line or of ErbB2 receptor in the Fischer rat 3T3 cell line (Fig. 4b), whereas our positive control, betacellulin[28], stimulated these receptors. On the other hand, rNRG-2β stimulated ErbB4 receptor in the Ba/F3 (ErbB4) cell line, as well as ErbB2 and ErbB3 receptors in the Ba/F3 (ErbB2 + ErbB3) cell line. These results indicate that neuregulin-2β signalling results from direct interaction with ErbB3 and/or ErbB4 receptors.

We have shown that the neuregulin-2 gene, which has structural similarity to the neuregulin gene, encodes new ligands for the ErbB3 and ErbB4 receptors. The distinct expression pattern of neuregulin-2 suggests that these proteins have specific biological functions. It will be necessary to compare neuregulin-2 with neuregulins and other ligands for ErbB-family receptors, including the temporal and spatial regulation of their expression, in order to understand the function of this multiligand/multireceptor signalling network.

Methods

Cloning of neuregulin-2 cDNAs. Two pools of degenerate oligonucleotides were synthesized based on two conserved regions of the neuregulin sequences, one in the immunoglobulin-like domain and the other in the EGF-like domain. Phages from an adult rat cerebellum cDNA library (gift from D. Zhao) were used as templates for PCR. Two steps were used to reduce neuregulin sequences and select neuregulin-related sequences. First, PCR products were digested with *Bcll* and separated by agarose gel electrophoresis, because there is a *Bcll* site in rat neuregulin cDNA[4]. DNA fragments of expected sizes were isolated from the agarose gel and reamplified with the same primers. Final PCR products were subcloned into pBlueScriptII vector (Stratagene). Second, individual clones were hybridized with a neuregulin probe under low-stringency conditions and positive clones were sequenced. We identified one clone, n9, which has significant homology to neuregulins. ^{32}P-labelled probes from the n9 insert were used to screen the cDNA library (~500,000 clones) and several positive clones were identified. The insert of each clone was sequenced in both directions and analysed.

Northern blot and *in situ* hybridization. Poly(A)$^+$ RNA was purified from tissues by using a FastTrack kit (Invitrogen). RNA samples were separated on agarose gels and transferred to nylon filters by standard procedures. Filters were hybridized with ^{32}P-labelled probes under high-stringency conditions. A probe was generated by random priming of a fragment of neuregulin-2 cDNAs spanning the EGF-like plus the Ig-like domains. The highly conserved transmembrane domain and adjacent sequence were excluded. The probe would hybridize to both neuregulin-2α and neuregulin-2β transcripts. *In situ* hybridization was done essentially as described[29]. We used a digoxigenin-labelled cRNA probe spanning the EGF-like plus the Ig-like domains. Several other probes derived from different parts of neuregulin-2 cDNAs (that is, the EGF-like domain only, the Ig domain only) also gave essentially the same hybridization pattern.

Expression of recombinant neuregulin-2 proteins. The insert of a partial neuregulin-2β cDNA clone was subcloned into the pRc/CMV expression vector (Invitrogen) and stably transfected into CHO cells. Serum-free conditioned medium was collected. Negative control media were conditioned medium from CHO cells or from CHO cells transfected with an unrelated gene. For expression of rNRG-2β in *E. coli*, the EGF-like domain of neuregulin-2β (residues 240–305) was subcloned into pQE32 expression vector with an N terminus 6 × histidine tag (Qiagen). Protocols for solubilization and refolding of proteins from inclusion bodies were essentially as described[24], except that refolded proteins were not purified further. The final rNRG-2β protein concentration is ~500 μg ml^{-1}.

Tyrosine-phosphorylation assay. MDA-MB 453 and T47D cells were starved in serum-free medium for 2–6 h before addition of neuregulin 2β conditioned medium, negative control medium, or heregulin-β1 (extracellular portion, 20 ng ml^{-1}; from S. J. Burden). After 5–10 min at room temperature, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1 SDS, 1 mM sodium orthovanadate, 50 μg ml^{-1} aprotinin, 0.5 mM PMSF), immunoprecipitated with rabbit antibodies (Santa Cruz Biotechnology) specific for ErbB2 (C-18), ErbB3 (C-17) or ErbB4 (C-18). Immunoprecipitated proteins were collected on protein A–Sepharose beads, analysed by western blotting with an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). Antibody binding was detected by enhanced chemiluminescence (Amersham Life Science). The recombinant Ba/F3 cell lines expressing ErbB family receptors have been described, as have protocols for stimulating and analysing ErbB-family receptor tyrosine-phosphorylation[2,26,27]. EGFR and ErbB2 expression in Fischer rat 3T3 (FR3T3) was described[28]. Human recombinant betacellulin (R&D Systems) was used at 200 ng ml^{-1}. Chemically synthesized heregulin-β1 65-mer[24] was used at 94 ng ml^{-1}. Although we can detect the activity of rNRG-2β at a dilution of 1:50,000, it was routinely used at a dilution of 1:100 to ensure saturated receptor phosphorylation.

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Supplementary information is available on *Nature*'s World-Wide Web site (http://www.nature.com) or as paper copy from Mary Sheehan at the London editorial office of *Nature*.

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Correspondence and requests for materials should be addressed to H.C. (e-mail: hanchang@leland.stanford.edu).

Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases

Kermit L. Carraway III*, Janet L. Weber†, Michelle J. Unger*, Jessica Ledesma†, Naichen Yu†, Martin Gassmann†§ & Cary Lai†

* *Department of Cell Biology, Harvard Medical School, and Division of Signal Transduction, Beth Israel Hospital, Boston, Massachusetts 02215, USA*
† *Department of Neuropharmacology, The Scripps Research Institute, La Jolla, California 92037, USA*
‡ *Molecular Neurobiology Laboratory, The Salk Institute, La Jolla, California 92037, USA*

The neuregulins (NRGs) are a family of multipotent epidermal-growth-factor-like (EGF-like) factors that arise from splice variants of a single gene. They influence the growth, differentiation, survival and fate of several cell types. We have now discovered a set of new neuregulin-like growth factors, which we call neuregulin-2 (NRG-2): these are encoded by their own gene and exhibit a distinct expression pattern in adult brain and developing heart. Like NRG-1, the EGF-like domain of the new ligands binds to both the ErbB3- and ErbB4-receptor tyrosine kinases. However, NRG-2 stimulates different ErbB-receptor tyrosine-phosphorylation profiles from NRG-1. Our results indicate that NRG-1 and NRG-2 mediate distinct biological processes by acting at different sites in tissues and eliciting different biochemical responses in cells.

The neuregulins (NRG-1) are a family of polypeptide growth factors that are thought to be critical for the developing heart and nervous system, and in the generation and progression of tumours. In the peripheral nervous system, neuronally derived NRG-1 acts at the neuromuscular junction to promote the end-stage differentiation of muscle cells[1–4], and in developing nerves to promote the proliferation, survival and differentiation of Schwann cells[5–6]. NRG-1 is also essential in the developing heart: neuregulin-deficient mice fail to form ventricular trabeculae and die in mid-gestation[7–10]. NRG-1 may also play a role in oncogenesis as it can indirectly activate the ErbB2-receptor protein-tyrosine kinase (PTK) whose overexpression correlates with a poor prognosis of some cancer patients[11]. The molecular cloning of these bioactivities has revealed that NRG-1, separately identified as glial growth factors (GGFs), acetylcholine-receptor-inducing activity (ARIA), heregulins (HRGs), and neu differentiation factor (NDF), are the alternatively spliced products of a single gene[12–15]. NRG-1 binds to ErbB3 and ErbB4 (refs 16–19), two members of the ErbB subfamily of receptor PTKs, and stimulates the tyrosine phosphorylation of other ErbB receptors through receptor heterodimerization[19–22].

We have identified a new NRG-1-like gene called neuregulin (NRG-2). Three distinct complementary DNAs were obtained by screening an adult mouse brain cDNA library with a NRG-1 probe; a composite amino-acid sequence is presented in Fig. 1a. NRG-2 is distantly related to NRG-1 (~35% identity) but exhibits a similar overall domain structure. It includes a single EGF-related motif with the same spacing between the third and fourth cysteine residues as that for EGF (and related molecules that bind to the EGF receptor) but distinct from NRG-1 (see Supplementary Information). NRG-2 carries a single immunoglobulin (Ig)-like domain that is ~36% identical to similar domains found in a subset of NRG-1 splice variants. The transmembrane segment is highly conserved (Fig. 1a, b) and, by analogy with NRG-1, cleavage of the NRG-2 precursor may yield a soluble NRG-2 isoform. The structure of the NRG-2 cDNAs indicates that alternative splicing similar to those found in NRG-1 (refs 12, 14) also generates multiple NRG-2 EGF-like isoforms, including a β1-like (clone 16A) and an α-like form (clone 5). The splicing alters the carboxy-terminal portion of the EGF-like motif; the NRG-2 and NRG-1 β1-like forms are particularly highly conserved in this region, with 16/28 identical residues.

To identify sites of NRG-2 expression we analysed, by northern blotting, messenger RNA samples from a series of adult rat tissues (Fig. 2a). Neural expression of NRG-2 mRNA was observed primarily in the cerebellum and olfactory bulb; a transcript was also detected in liver. By whole-mount *in situ* hybridization in mouse E9.5 embryos, NRG-2 mRNA was detected in the endothelial lining of the heart, with the highest mRNA levels being found in the atrium and lower levels in the ventricle and outflow tract (Fig. 2b, c). This profile is complementary to that of NRG-1, which is expressed at high levels in the ventricular endothelium and only weakly in the atrial endothelium[7]. It also differed from NRG-1 in the developing hindbrain, where NRG-1 has been identified in rhombomeres 2, 4 and 6 (ref. 7) and NRG-2 was not detected. In the adult rat, expression of NRG-2 and NRG-1 mRNA was compared by *in situ* hybridization. In the cerebellum, NRG-2 mRNA was detected in granule and Purkinje cells (Fig. 2k), whereas NRG-1 was found mainly in Golgi II cells[23,24] (Fig. 2j). Cerebellar granule cells also express ErbB4 mRNA (C.L., unpublished results), suggesting a possible autocrine role for NRG-2. In the hindbrain, the NRG-2 mRNA was weakly detectable in the motor trigeminal nucleus (Fig. 2i), compared to a strong NRG-1 signal (Fig. 2h). In the hippocampus, granule cells of the dentate gyrus express NRG-2 (Fig. 2g), where no NRG-1 was detected (Fig. 2f). The cholinergic cells in the basal forebrain that express NRG-1 mRNA[23,24] (Fig. 2d) are only faintly positive for NRG-2 (Fig. 2e). NRG-2 mRNA was not detected in the hypothalamus[23,24], which produces the pituitary-derived NRG-1 known as GGF (not shown). These profiles indicate that in the adult brain NRG-1 and NRG-2 are expressed in largely non-overlapping neural cell populations, suggesting that the two ligands have distinct functional roles.

§ Present address: Division of Neurobiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.